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Product Purification by Reversible Phase Transition Following *Escherichia coli* Expression of Genes Encoding up to 251 Repeats of the Elastomeric Pentapeptide GVGVP

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By constructing a basic gene unit encoding (GVGVP)₁₀, it was possible to build concatemer genes with as many as 25 repeats of the monomer unit encoding a protein-based polymer with a molecular weight of greater than 100,000 Da. This employed the use of terminal cloning adaptor oligonucleotides as chain terminators to enhance the desired polymer gene size distribution. These genes have been expressed in *Escherichia coli* and the products have been purified from the culture lysates using a simple centrifugation method which relies upon the inverse temperature transitional properties of these elastomeric protein-based polymers. At 4°C, the polymers are soluble; on raising the temperature above 26°C, the onset temperature (*T*_i) for the (GVGVP)₂₅₁ inverse temperature transition, the polymer separates out as the more dense phase. Upon shifting the temperature between 4 and 37°C, the recombinant elastomeric protein-based polymers undergo reversible phase transitions from soluble (4°C) to insoluble (37°C) allowing their separation from other cellular components by several cycles of centrifugations at alternate transitional states. Additionally, centrifugation, at a temperature just below *T*_i, allows for dramatic lowering of endotoxin levels. Furthermore, many ways of varying the value of *T*_i, such as adding salt to lower *T*_i or changing the degree of ionization in polymers with functional side chains, can be used to achieve purification of more complex polymers. © 1996 Academic Press, Inc.

The synthesis of peptide-based polymers, with different biophysical properties resulting from variations in

sequences, can provide polymers that contribute to an understanding of the parameters that guide protein folding and function and can provide materials with a wide range of applications. A primary advantage of using peptide-based polymers for these purposes is the ability to study a model protein with a relatively simple composition and then to build steadily on the knowledge gained from the preparation and characterization of polymers with systematic and ever more complex variations. One element of our work in this area has utilized the chemical synthesis and characterization of polypeptides of the structure poly[*f*_v(GVGaP)*f*_x(GX-GaP)], where *f*_v and *f*_x are mole fractions with *f*_v + *f*_x = 1, where X is any of the naturally occurring amino acid residues, and where α is V, I, or F (1-4). Another element of our work has involved the chemical synthesis of fixed sequence polypentadecapeptides and polytricosapeptides for physical characterization and for the development of principles of protein folding and function in energy conversion (5-8) and in applications, for example, such as drug delivery vehicles (9). Accordingly, chemical syntheses have been particularly useful for generating moderate amounts of the above model proteins in a timely fashion in order to survey ranges of properties and applications.

However, chemical synthesis as a practical method has been pushed to its limit as the required amount and complexity of the polymers continues to increase. For this reason and in the long run to reduce cost, we have increasingly utilized a genetic engineering approach to achieve the synthesis of these substances. This biosynthetic approach offers the potential for long polymers of defined length without the racemization and impurity problems that plague chemical synthesis

and with various functional residues placed in the sequence with the desired structural relationship maintained throughout the polymer (10). Once a gene is constructed, biosynthesis does not require a complex, multistep synthesis with difficult quality control for each batch that is produced, and it promises an economy of scale that is currently unobtainable by means of chemical synthesis.

Others have reported the construction and expression of genes encoding tandem repeating peptides in *Escherichia coli* (11–15). Our own efforts at the biosynthetic route to produce high molecular weight peptide-based polymers have focused initially upon the synthesis and expression of genes encoding poly(GVGVP) elastomers. We have previously demonstrated the biosynthesis of (GVPGV)₂₀ (16) in *E. coli* as a fusion to glutathione S-transferase (*gst*). This fusion protein was purified by chromatography based on the affinity of the *gst* moiety for glutathione attached to a solid support (17). The gene was constructed using a modular approach whereby a DNA sequence encoding 10 repeats of the pentamer, (GVPGV)₁₀, was first synthesized and cloned as an in-frame addendum to the *gst* gene. Following this, another copy of the 10-mer gene was cloned behind the first as a tandem repeat to create the 20-mer gene fused to the *gst* gene. This modular approach allowed demonstration of the capacity to construct, express, and have stably maintained a gene encoding multiple repeats of the pentamer in *E. coli*. Subsequently, a concatemeric route has been pursued to achieve the synthesis of elastomeric peptide-based polymer genes encoding poly(GVGVP) resulting in genes with 41, 121, 141, and 251 repeats of the pentamer sequence. This has involved the construction of a basic gene unit (catemer) encoding 10 repeats of the GVGVP peptide which could be enzymatically self-ligated through nonpalindromic single-stranded extensions to produce multiple tandem repeats of the basic gene. In addition, double-stranded synthetic oligonucleotides are added to the concatenation ligation reactions not only to provide the terminal restriction endonuclease recognition sites required for cloning the concatemer genes, but also as "chain terminators" to enhance the amount of polymer product in a desired size range.

The above poly(GVGVP) genes have been expressed to high levels in *E. coli* using a T7 promoter expression system (18) and, as shown here, are easily purified from the cellular lysate to a high degree of purity by a simple centrifugation procedure. This is made possible due to the particular inverse temperature transition behavior of these elastomeric polypeptides (1,19) which causes them to undergo a reversible phase transition from soluble to insoluble upon increasing the temperature or upon variously lowering the transition temperature from above to below the operating temperature. The

expressed recombinant GVGVP polymers are soluble in the cellular lysate at low temperatures at which point the normally insoluble constituents can be removed by centrifugation. Then, upon raising the temperature to, for instance, 37°C the protein-based polymer comes out of solution to form a highly visible aggregate which can also be removed from the remaining soluble constituents by centrifugation. Several repeats of this cycle following solubilization of the model protein in cold solution results in an extremely high degree of purification as seen by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and nuclear magnetic resonance (NMR) analyses. Furthermore, temperature-dependent aggregation can also be used to decrease the protein-based polymer pyrogenicity due to the presence of *E. coli* cell wall fragments. In particular, additional centrifugation at a temperature just below the *T_i* value for the protein-based polymer becomes an important step in critically lowering the endotoxin levels toward those required for medical applications.

MATERIALS AND METHODS

Gene Constructions and Cloning

PCR (20) was performed using Taq DNA polymerase purchased from Life Technologies, Inc. (LTI; Gaithersburg, MD) and synthetic oligonucleotides from Oligos, Etc. (Wilsonville, OR). The 5' oligonucleotide (oligo) had the sequence CTGATCGAAGGATCCAGGCGT-TGGTGTA and the 3' oligonucleotide (reading from the 5' to the 3' end) had the sequence GTCAGTCAGTCA-GGATCCAACGCCTGGAACACCTACA. Cloning and molecular biology techniques were performed using standard procedures (21); enzymes *Bam*HI, *Nco*I, T4 DNA ligase, and T4 kinase were from LTI and *P*fMI was from New England Biolabs (Beverly, MA). Plasmid pUC118 was from United States Biochemicals (USB; Cleveland, OH) and was used for initial gene cloning before subcloning into the expression plasmid pET-11d from Novagen, Inc. (Madison, WI). DNA sequencing was performed using the universal primer and Sequenase kit from USB. *E. coli* strain DH5aF' (*lacZ*ΔM15, *recA*1) from LTI was used as the host for gene clonings, and strain GM2969 (*dcm*6, *recA*56), the kind gift of Dr. M. G. Marinus (U. Mass., Worcester, MA), was used to prepare plasmid with nonmethylated *P*fMI sites for isolation of the catemer gene fragments. The *P*fMI gene fragments were isolated by electroelution following electrophoresis through a 6% polyacrylamide gel. The adaptor oligos were purchased from Cruachem (Dulles, VA) and had the sequences shown in Fig. 1B. The concatenation ligation reactions were performed in duplicate with the catemer gene at a concentration of 1.0 μM and with the 5' adaptor oligos in one reaction and the 3' adaptors in the other, each at a concentra-

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tion of 0.135 μM . The ligations were allowed to proceed for 1 h at 23°C after which time the two reactions were mixed and continued for another 2.5 h. The ligation products were size fractionated by electrophoresis through a 1% agarose gel and extracted by either electroelution or GeneClean (BIO 101, Inc., Vista, CA).

Protein Expression and Purification

E. coli hosts used for expression were BL21(DE3) and/or HMS174(DE3) (*recA1*) both from Novagen, Inc. Cultures were grown at 37°C in LB medium (21) containing 100 $\mu\text{g}/\text{ml}$ ampicillin. Cell density was monitored using a Klett–Summerson colorimeter with a red No. 66 filter. Gene expression was induced by the addition of IPTG to 0.4 mM at a cell density of 90 klett units and cells were harvested by centrifugation following 3 h of continued growth. Cells were resuspended in water and lysed by sonic disruption. Insoluble debris was removed by centrifugation at 10,000g at 4°C for 20 min. An equal volume of 2 \times TN buffer (100 mM Tris–HCl, pH 8.0, 1.0 N NaCl) was then added to the cleared lysate and it was warmed to 37°C in a warm water bath to induce formation of the recombinant protein aggregate. The protein was then recovered by centrifugation at approximately 1,500g at 37°C and was resuspended and dissolved in cold water. Typically, the process of cold and warm centrifugation was repeated two more times with 2 \times TN added prior to the warm spin. Qualitative analysis of the product was performed by SDS–PAGE (22) followed by staining with 0.3 M CuCl_2 (23). Staining with copper creates a negative image by exclusion of the stain from the gel by the protein. Further purification by centrifugation, to remove endotoxins arising from cell membrane fragments, utilized a temperature immediately below the onset temperature, T_i , of the inverse temperature transition. The protein is stored either in water at 4°C or dry at room temperature following lyophilization.

Determination of T_i and Temperature-Induced Aggregation Profiles to Assess Purity

The temperature profiles for aggregation, which give rise to the T_i values, were determined using an AVIV-updated Cary Model 14 spectrophotometer. The wavelength was held at 300 nm; the turbidity was determined as the temperature was raised at a rate of 30°C per hour, and the sample resided within a 300 Hz vibrator in order to minimize settling. At low temperature, there is zero absorbance or turbidity, and, as the temperature is raised, for pure samples there occurs an abrupt increase in turbidity identifying the onset of the inverse temperature transition. The value of T_i is defined as the temperature at which 50% turbidity occurs.

NMR Characterization of Purified Poly(GVGVP)s

The proton magnetic resonance spectra were obtained at 400 MHz on a Bruker WH-400 spectrometer equipped with an Aspect 3000 computer for data work up. The sample was dissolved in dimethylsulfoxide- d_6 (DMSO) and the 2.5 ppm chemical shift was used as the reference. The temperature of the sample was 24°C; the 90° pulse width was 7.9 μs , and the spectra resulted from 32 pulses. Sample preparation utilized the centrifugations as described above followed by dialysis against deionized/distilled, low conductivity water, lyophilization, and dissolution in DMSO.

RESULTS AND DISCUSSION

Construction of the Catemer (GVGVP)₁₀ Gene

A gene comprising the unit catemer (Fig. 1A) and encoding (GVGVP)₁₀ with *Bam*HI and *Pfl*MI sites at both termini, (GVGVP)₁₀-BP, was constructed by PCR amplification utilizing as the template a (GVGVP)₁₀ gene that exists as a fusion to *gst* in pGEX-3X (Pharmacia). The fusion gene had previously been constructed by PCR (unpublished) utilizing another *gst* fusion construct, pEPP-2 (16), as the template resulting in the conversion of *gst*-(GVPGV)₁₀ to *gst*-(GVGVP)₁₀. The (GVGVP)₁₀-BP PCR product was digested with *Bam*HI and inserted into pUC118 resulting in plasmid pUC-(GVGVP)₁₀. Positive clones were verified by DNA sequence analysis.

Construction of the Concatemer Genes

Restriction endonuclease *Pfl*MI recognizes the interrupted palindrome CCANNNN'NTGG and cleaves at the position designated by the apostrophe to leave 3-base 3' single-stranded extensions. These ends are not selfcomplementary like those generated by cleavage at, for instance, a 6-base palindromic restriction endonuclease recognition site. Therefore, preparation of the basic gene units by digestion with an enzyme like *Pfl*MI results in fragments that, upon selfligation, can only go together in a head-to-tail fashion, thereby maintaining the correct reading polarity for subsequent translation. A group of restriction enzymes that should be of more general utility to generate gene fragments for concatenation are those that cleave outside of their recognition site since these sites could be placed flanking the catemer gene sequences.

The *Pfl*MI recognition site is blocked from cutting when methylated by DNA cytosine methylase (*Dcm*); therefore, it was necessary to grow pUC-(GVGVP)₁₀ in the *dcm* strain GM2969 for the purpose of preparing large amounts of the *Pfl*MI catemer gene fragment. After purifying substantial amounts of the *Pfl*MI gene fragment, a series of ligation reactions was performed with increasing amounts of the gene fragment to deter-

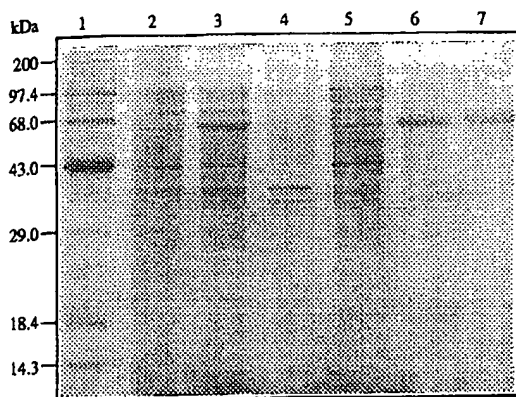


FIG. 2. A copper stained SDS-polyacrylamide gel with samples taken from expression through purification of (GVGVP)₄₁. Lane 1, molecular weight marker; 2, preinduction whole cell lysate; 3, 3-h postinduction whole cell lysate; 4, cold-spin pellet; 5, warm-spin supernatant; 6, warm-spin pellet; 7, third warm-spin pellet. Pre- and postinduction samples were loaded at 4 klett/ml per lane and subsequent samples were normalized to this amount.

utes in one's hand causes the appearance of an opaque aggregate; reinserting the tube into the ice causes an almost immediate clearing. Exploiting this characteristic allowed the purification of these proteins by a procedure involving selective centrifugation. First the cellular lysate was centrifuged at a low temperature (4°C) to remove insolubles and cellular debris. Then the cleared lysate was warmed to 37°C to effect the poly(GVGVP) phase transition and aggregation which was also then removed from the soluble component by centrifugation. The recovered polymer protein was then resuspended and solubilized at the low temperature. This single round of cold and warm centrifugation achieves a remarkable degree of purification, although the process was typically repeated two more times. Figure 2 shows an SDS-polyacrylamide gel with samples taken from the expression through the purification steps for the (GVGVP)₄₁. As one might expect, expression of the polymer proteins at 37°C should result in their subsequent aggregation *in vivo*, and in fact, visualization of postinduction cells under the light microscope reveals the presence of refractile inclusion bodies. However, the intracellular aggregate is apparently dissolved following sonic disruption of the cells at 4°C.

Figure 3 shows the final purification products from lab-scale fermentations for the 41, 121, 141, and 251 repeats of (GVGVP). We have routinely used copper staining of SDS-PAGE gels to visualize these proteins because they stain so poorly by Coomassie blue or silver. This characteristic also makes them difficult to quantitate by the more commonly used stain binding protocols. When staining the gels with copper chloride, we frequently observe the poly(GVGVP) proteins as an opaque white band on the gel; in particular, the 41 mer

almost always appears this way as, for example, is seen in Fig. 3. Notice also that the 41 mer runs with an apparent molecular weight larger than the calculated molecular weight. The gene DNA fragment, however, sizes against the ligation ladder and other molecular weight markers as a 41 mer.

The 400 MHz proton nuclear magnetic resonance spectrum for (GVGVP)₂₅₁ is given in Fig. 4 and compared to that of the chemically synthesized poly(GVGVP). As noted in Fig. 4, the T_1 for (GVGVP)₂₅₁ is just slightly lower than that of poly(GVGVP), suggesting that the latter is of a slightly higher molecular weight as confirmed by SDS-PAGE analysis. The two spectra are virtually identical. The very small peaks near 3.5, 4.5, and 7.7 ppm in both spectra are due to an approximate 5% of the *cis* isomer at the Val-Pro bond. It is abundantly clear that (GVGVP)₂₅₁ has been prepared and purified to a high level. Similar NMR data have been obtained verifying the other lower molecular weight gene products, with the interesting feature that the spectrum for (GVGVP)₄₁ exhibited an additional Val γCH_3 resonance which was less than 1.5% (a best estimate of 1.2%) of the primary Val γCH_3 peak. This is reasonably interpreted as an end effect due to one of the 82 Val residues, and it is consistent with an n of 41.

When using a 500-liter fermentation, the same procedure, as described above for centrifugation at 4°C and then at 37°C, resulted in the temperature profile for aggregation of Fig. 5 with the shoulder between 20 and 25°C. This demonstrates the sensitivity of the profile to the presence of impurities. If centrifugation is then carried out at 23°C, the precipitate removed, and the temperature profile for aggregation again determined,

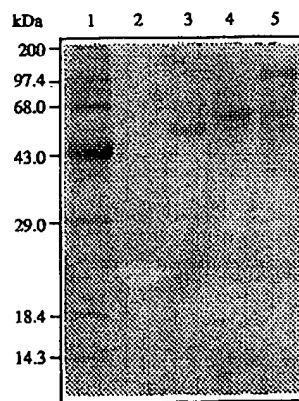


FIG. 3. A copper stained SDS-polyacrylamide gel showing purified GVGVP peptide polymers after three cycles of cold and warm centrifugations. The polymers with their calculated molecular weights given in parenthesis are: lane 1, molecular weight markers; 2, 41 mer (16,787); 3, 121 mer (49,507); 4, 141 mer (57,687); and 5, 251 mer (102,677). The 41 mer runs with an apparent molecular weight larger than that calculated (see text).

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